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ENVIRONMENTAL MICROBIOLOGY

Blame It on the Metabolite: 3,5-Dichloroaniline Rather than the Parent Compound Is Responsible for the Decreasing Diversity and Function of Soil Microorganisms

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ABSTRACT Pesticides are key stressors of soil microorganisms with reciprocal effects on ecosystem functioning. These effects have been mainly attributed to the parent compounds, while the impact of their transformation products (TPs) has been largely overlooked. We assessed in a meadow soil (soil A) the transformation of iprodione and its toxicity in relation to (i) the abundance of functional microbial groups, (ii) the activity of key microbial enzymes, and (iii) the diversity of bacteria, fungi, and ammonia-oxidizing microorganisms (AOM) using amplicon sequencing. 3,5-Dichloroaniline (3,5-DCA), the main iprodione TP, was identified as a key explanatory factor for the persistent reduction in enzymatic activities and potential nitrification (PN) and for the observed structural changes in the bacterial and fungal communities. The abundances of certain bacterial (*Actinobacteria*, *Hyphomicrobiaceae*, *Ilumatobacter*, and *Solirubrobacter*) and fungal (*Pichiaceae*) groups were negatively correlated with 3,5-DCA. A subsequent study in a fallow agricultural soil (soil B) showed limited formation of 3,5-DCA, which concurred with the lack of effects on nitrification. Direct 3,5-DCA application in soil B induced a dose-dependent reduction of PN and NO_3^--N, which recovered with time. *In vitro* assays with terrestrial AOM verified the greater toxicity of 3,5-DCA over iprodione. "*Candidatus* Nitrosotalea sinensis" Nd2 was the most sensitive AOM to both compounds. Our findings build on previous evidence on the sensitivity of AOM to pesticides, reinforcing their potential utilization as indicators of the soil microbial toxicity of pesticides in pesticide environmental risk analysis and stressing the need to consider the contribution of TPs in the toxicity of pesticides on the soil microbial community.

IMPORTANCE Pesticide toxicity on soil microorganisms is an emerging issue in pesticide risk assessment, dictated by the pivotal role of soil microorganisms in ecosystem services. However, the focus has traditionally been on parent compounds, while transformation products (TPs) are largely overlooked. We tested the hypothesis that TPs can be major contributors to the soil microbial toxicity of pesticides using iprodione and its main TP, 3,5-dichloroaniline, as model compounds. We demonstrated, by measuring functional and structural endpoints, that 3,5-dichloroaniline and not iprodione was associated with adverse effects on soil microorganisms, with nitrification being mostly affected. Pioneering *in vitro* assays with relevant ammonia-

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oxidizing bacteria and archaea verified the greater toxicity of 3,5-dichloroaniline. Our findings are expected to advance environmental risk assessment, highlighting the potential of ammonia-oxidizing microorganisms as indicators of the soil microbial toxicity of pesticides and stressing the need to consider the contribution of TPs to pesticide soil microbial toxicity.

KEYWORDS 3,5-dichloroaniline, ammonia-oxidizing archaea, ammonia-oxidizing bacteria, iprodione, pesticide transformation products, soil microbial toxicity

The toxicity of pesticides on nontarget organisms, including pollinators and aquatic and terrestrial organisms, is well documented (1–4). In the last 10 years, much attention has also been given to the effects of pesticides on soil microorganisms, considering their pivotal role in ecosystem functioning (5). Several studies have used advanced molecular and biochemical tools to assess the toxicity of pesticides in relation to the structure and function of the soil microbial community (6–8). The majority of them have considered the parent compound as the causal agent of the toxic effects observed, while the potential toxicity of transformation products (TPs) has attracted little attention. Recently, Karas et al. (9) demonstrated the major role of TPs of chlorpyrifos and isoproturon on the inhibition of soil microbial functions. Similarly, Papadopoulou et al. (10) showed that quinone imine, the oxidized TP of ethoxyquin (an antioxidant used in the fruit-packaging industry) was equally as toxic as, or more toxic than, the parent compound to ammonia-oxidizing microorganisms (AOM). This group has been shown to be particularly sensitive to environmental perturbations (11) including pesticide exposure (6, 9).

Iprodione is a fungicide used for the control of foliage and soilborne fungal pathogens (12). It is a potential carcinogen (13) and an endocrine-disrupting substance (14). Microbial degradation is the main process controlling its dissipation in soil and leads to the formation of 3,5-dichloroaniline (3,5-DCA) (15). 3.5-DCA is neurotoxic (16), and it is considered the most recalcitrant (17) and toxic (18) dichloroaniline isomer. Previous studies have reported significant reductions in the abundance, activity, and diversity of soil microorganisms in response to iprodione application (19–21). However, the effects observed were attributed entirely to the parent compound, while the transformation of iprodione in soil and the potential contribution of its TPs were not explored.

The main aim of this study was to assess the toxicity of iprodione on the soil microbial community and explore the role of 3,5-DCA for the potential adverse effects observed. This was achieved through a series of laboratory microcosm assays replicated in two different soils (to verify the uniformity of effects observed) where the dissipation of iprodione and the formation of 3,5-DCA were determined analytically and correlated with changes in (i) functional enzyme activity, (ii) the abundance of functional microbial groups, and (iii) the bacterial, fungal, and AOM community determined via high-throughput amplicon sequencing. Microbial taxa responsive to iprodione and 3,5-DCA were identified, and the toxicity of 3,5-DCA was further verified in soil microcosm tests with direct application of 3,5-DCA. The toxicity of iprodione and 3,5-DCA to AOM was confirmed using *in vitro* tests with isolated strains of terrestrial ammonia-oxidizing bacteria (AOB) and archaea (AOA).

RESULTS

Dissipation, transformation, and impact of iprodione on the soil microbial community: microcosm assay in the meadow soil. We initially determined in a lab microcosm study the dissipation/transformation of iprodione and its impact (applied at 1, 10, and 100 times the recommended dose [1×, 10×, and 100× dose, respectively]) on the activity, abundance, and diversity of the soil microbial community in a meadow soil. The time required for 50% dissipation (DT_{50}) of iprodione in the different treatments was calculated with the single first-order (SFO) kinetic model, which showed the best fit to the measured data. The persistence of iprodione, as depicted by the

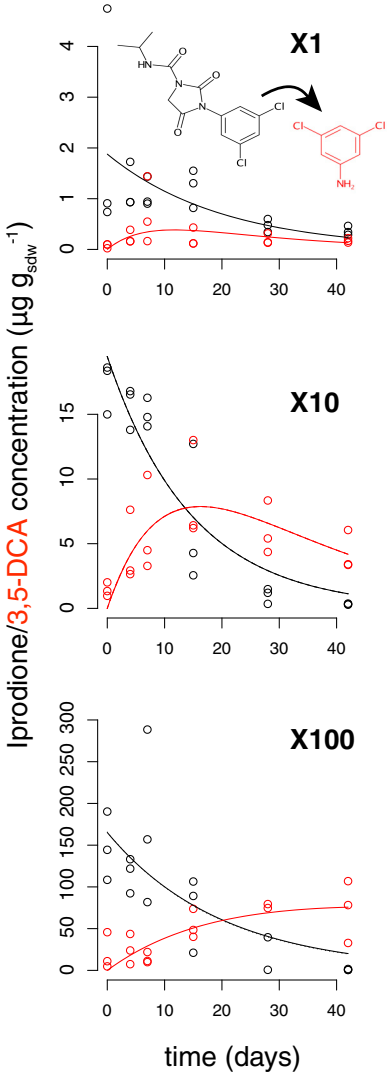

FIG 1 The dissipation of iprodione (black circle) and the formation of 3,5-dichloroaniline (3,5-DCA) (red circle) in soil samples treated with 1, 10, and 100 times the recommended dose. Dissipation data were fitted to the single first-order (SFO) kinetic model which provided the best fit to the dissipation data in all cases.

calculated DT_{50} values, did not follow a dose-dependent trend and ranged from 10.2 days in the samples treated with the 10× dose to 14.0 days in the samples treated with the 1× dose (Fig. 1 and Table S1 in the supplemental material). Iprodione was hydrolyzed to 3,5-DCA, which was partially degraded in samples treated with the 1× and 10× doses but accumulated in the samples treated with the 100× dose (Fig. 1).

The application of iprodione induced significant main effects on the activity of several enzymes (Fig. S1) and potential nitrification (PN) (Fig. 2). A temporal dose-dependent trend was evident only for leucine aminopeptidase (Leu), where the 100× dose reduced activity from 3 days onward (Fig. S1g). PN activity was also significantly reduced by iprodione application, with the 10× dose transiently decreasing PN at 3 and 15 days ($P < 0.05$) before recovery occurred at 28 days and with the 100× dose resulting in a persistent reduction in PN from 7 days onward (Fig. 2). Pearson's correlation testing identified significant negative correlations between the soil levels of iprodione and the activity of acid phosphomonoesterase (AcP) (−0.39, $P < 0.001$), beta-glucosidase (Bglu) (−0.32, $P < 0.01$), and Leu (−0.25, $P < 0.05$) (Table S2). However, stronger negative correlations were evident between the soil concentrations of 3,5-DCA, formed by the hydrolysis of the applied iprodione, and the activity of

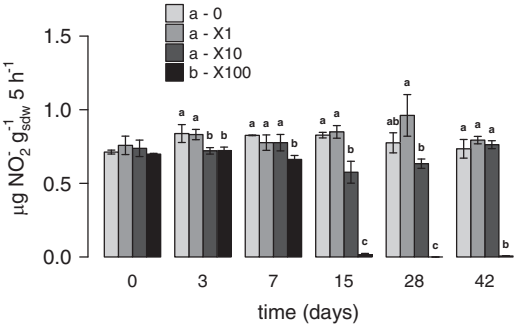

FIG 2 The potential nitrification (PN) rate in samples of soil A (meadow) which were either untreated (0, control) or treated with iprodione at 1, 10, and 100 times the recommended dose and collected at 0, 3, 7, 15, 28, and 42 days posttreatment. Each value is the mean of three replicates ± the standard deviation. Letters next to the dose descriptions (0, X1, X10, and X100) in the key provide the dose main-effect groupings according to the *post hoc* analysis. Within each time point, groups designated by the same letter are not significantly different at the selected α-value levels. sdw, soil dry weight.

phosphodiesterase (BisP) (-0.25, $P < 0.05$), *N*-acetyl-β-D-glucosaminidase (Chit) (-0.28, $P < 0.05$), Leu (-0.560, $P < 0.001$), pyrophosphatase (Piro) (-0.42, $P < 0.001$), and PN (-0.86, $P < 0.001$). Redundancy analysis (RDA) of measured enzymatic activities and PN using the ln-transformed soil concentrations of iprodione or 3,5-DCA as explanatory variables (Fig. 3) showed the following: (i) a weak but still significant effect ($P = 0.008$) of the concentrations of iprodione in soil, with treatment with the 100× dose clustering away from the other treatments along the iprodione concentration gradient, and (ii) a strong significant effect ($P = 0.001$) of the concentrations of 3,5-DCA in soil, with the samples treated with the 10× and 100× doses grouping away from samples treated with the 1× dose and the untreated samples along the 3,5-DCA concentration gradient. The application of iprodione affected the abundance of the *amoA* gene of AOA and the *soxB* gene of sulfur-oxidizing bacteria (SOB) (Fig. S2). However, the effects observed were either isolated to a single time point (i.e., at 0 days for the *amoA* gene of AOA where the samples treated with the 10× and 100× doses showed a significantly lower abundance than the control) or did not follow a dose-dependent pattern (i.e., the abundance of the *amoA* gene of AOA and of the *soxB* gene of SOB at 42 days was

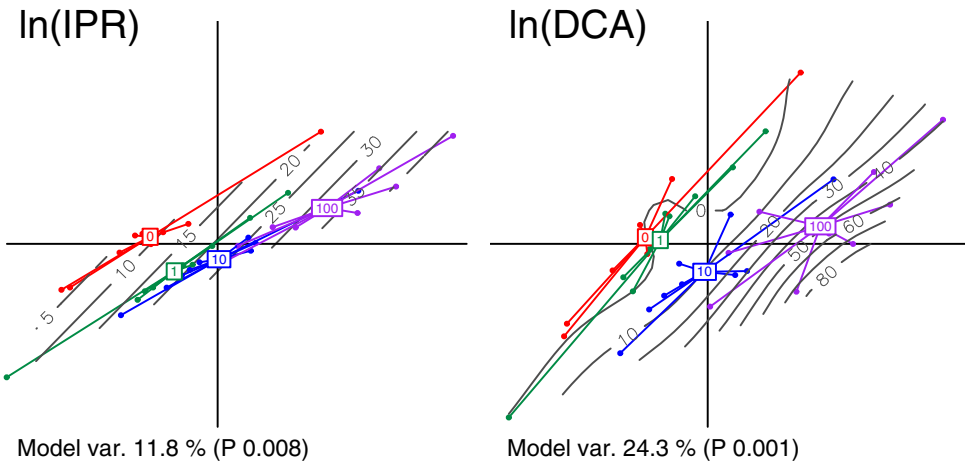

FIG 3 Redundancy analysis (RDA) on the enzymatic activities and potential nitrification data modeled along the ln-transformed measured soil levels of iprodione (IPR) and 3,5-dichloroaniline (3,5-DCA). The plots provide the information on the tested parameter (top left of each panel), the model shared variance and significance (999 permutations), and the plotted percentages of this variance along the axes. The sample points of the scatterplots are colored according to the dose, and 95% confidence ellipses are provided for each group along with the labeled centroid. In the case of continuous tested parameters, the contour lines show the parameter gradients.

significantly lower in the samples treated with the 1× dose than in samples treated with the 10× dose).

The effect of iprodione and 3,5-DCA on the α- and β-diversities of soil bacteria, fungi, and AOM was also assessed. Good's coverage estimate for bacteria, fungi, AOB, and AOA had mean values of 82.9, 99.8, 97.1, and 99.7%, respectively, suggesting good coverage of the bacterial (dominant), fungal, and AOM operational taxonomic unit (OTU) diversity by the devoted sequencing effort. Average richness values of 7,710, 1,491, 810, and 149 OTUs were observed in all control samples for the total bacterial 16S rRNA (at 97% sequence identity, used for OTU definition), fungal internal transcribed spacer (ITS; 97% identity), and AOB *amoA* and AOA *amoA* genes (99% identity for both *amoA* population OTUs), respectively. No major effects of iprodione or 3,5-DCA on the α-diversity were observed (Table S3).

Hierarchical clustering analysis of the taxonomy affiliations of the soil microorganisms revealed no grouping of the samples according to the applied doses or time for any of the microbial groups studied (Fig. S3). *Actinobacteria*, *Proteobacteria* (*Alpha-*, *Beta-*, *Gamma-*, and *Deltaproteobacteria*), and bacilli dominated the bacterial community in all soil samples, while the fungal community was dominated by unclassified fungi, *Ascomycetes*, *Basidiomycetes*, and members of the family *Mortierellaceae*. Representative sequences of AOA and AOB, according to their abundances in each OTU, were selected and placed in previously generated phylogenetic trees (22, 23). The vast majority of AOB sequences were placed within *Nitrosospira* clades with the most abundant OTUs (OTU00001, OTU00002, and OTU00005, encompassing 49% of the analyzed sequences) residing in *Nitrosospira* sp. strains Nsp17 and Nsp2. The sister clade of the *Nitrosospira multiformis*/Monterey Bay C clade contained other dominant OTUs (OTU00004, OTU00006, and OTU00009) (Fig. S4). Other highly abundant OTUs resided in the *Nitrosospira briensis* (OTU00003), *Nitrosospira* sp. strain Nsp 12 (OTU00007), and *Nitrosospira* sp. strain Nsp 65 (OTU00008) clades. These OTUs all together accounted for 85% of the total number of analyzed sequences. AOA were dominated by OTUs whose representative sequences were mainly placed in the *Nitrososphaerales* γ, δ, and ε clades (Fig. S5). The most dominant OTUs, accounting for 67.7% of the total number of analyzed sequences (OTU0001, OTU0004, and OTU00005), were placed in the *Nitrososphaerales* ε-2.2. clade, followed by OTUs belonging to different subgroups of the *Nitrososphaerales* γ clade (OTU0003, OTU0006, OTU0007, OTU0008, and OTU0009), accounting for 16.1% of the total number of sequences, and OTUs of the *Nitrososphaerales* δ clade (OTU0002, OTU0010, and OTU0013), accounting for 12.5% of the total number of analyzed sequences.

Partial RDA (not considering the time factor) of OTUs of the different microbial groups with the measured soil concentrations of iprodione (Fig. S6a) and 3,5-DCA (Fig. S6b) as explanatory variables indicated that only the latter had a significant effect on the structure of the bacterial ($P = 0.002$) and fungal ($P = 0.019$) communities. Pearson's correlation testing showed a significant negative correlation between the soil levels of 3,5-DCA and the abundance of *Actinobacteria*, *Hyphomicrobiaceae*, *Ilumatobacter*, and *Solirubrobacter* and a positive correlation with *Arthrobacter* (Fig. 4). Conversely, iprodione soil concentrations were positively correlated with *Arthrobacter*, *Marmoricola*, and *Xanthomonadaceae*. Analysis of fungal ITS sequences revealed that 3,5-DCA concentrations were negatively correlated with *Pichiaceae* and positively correlated with *Lasiosphaeriaceae*, while iprodione showed positive correlations with *Sordariomycetes*. 3,5-DCA and iprodione soil levels showed negative correlations with the less dominant AOB OTUs like OTU00016 (Nsp65 group), OTU00031, and OTU00038 and positively correlated with OTU00051, all belonging to the *N. briensis*/*N. multiformis*/Monterey Bay C group. Certain AOB OTUs were affected only by 3,5-DCA (OTU00034 negatively) or iprodione (OTU00058 negatively; OTU00042 positively). For AOA, 3,5-DCA concentrations were positively correlated with dominant OTUs like OTU00003 and OTU00009 (residing in the *Nitrososphaerales* γ-1.2 clade), while iprodione concentrations were negatively correlated with OTU00005 (residing in the *Nitrososphaerales* ε-2.2 clade), one of the most dominant AOA OTUs.

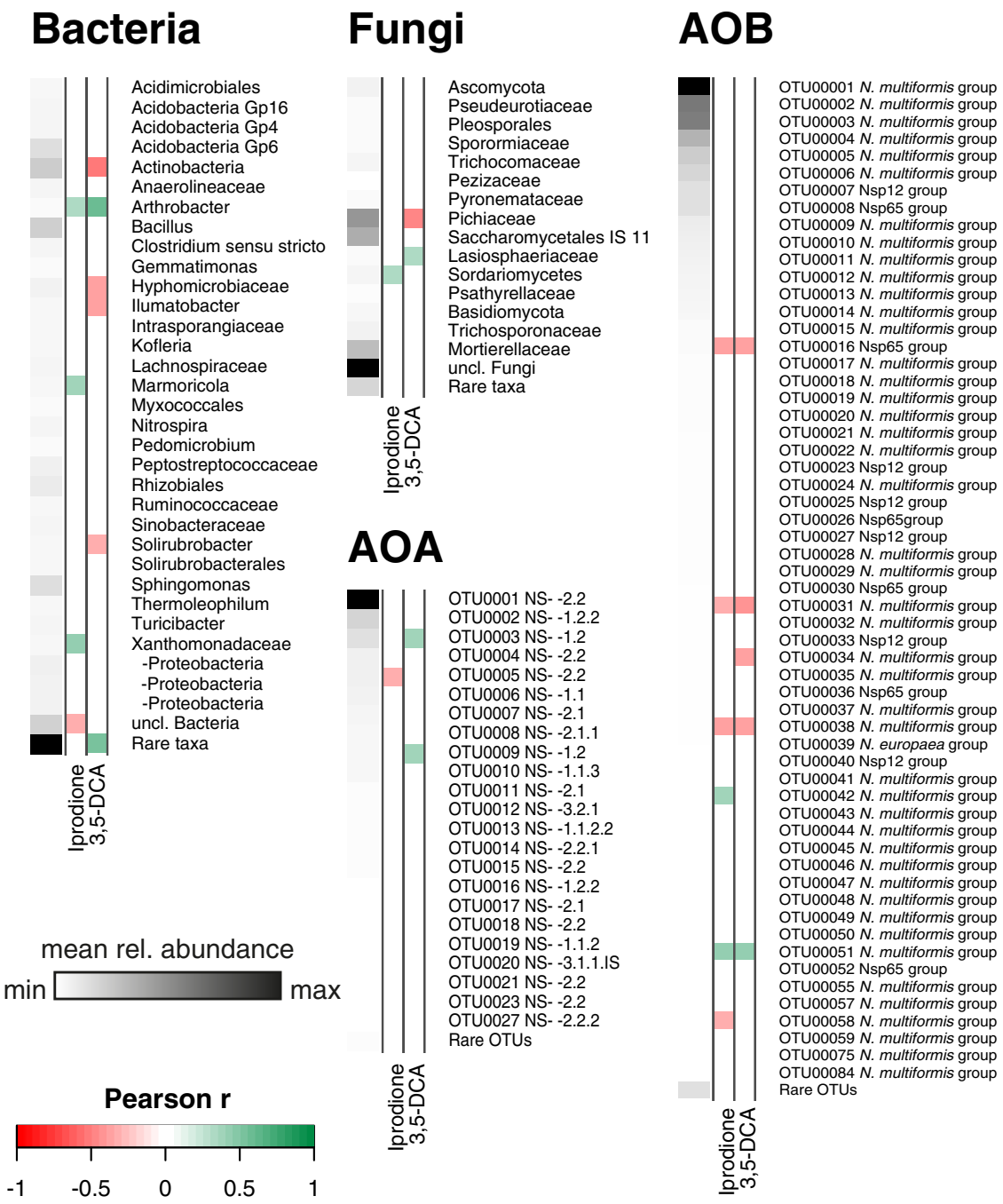

FIG 4 Heat map of Pearson correlations between the ln-transformed measured concentrations of 3,5-dichloroaniline (3,5-DCA) and iprodione (IPR) in soil and bacterial phyla/taxa/families, fungal phyla/taxa/families, and OTUs of ammonia-oxidizing bacteria (AOB) and archaea (AOA). The left-hand side gray-scale boxes are indicative of the mean relative abundances of these OTUs/taxa throughout the data set. *N. europaea*, *Nitrosomonas europaea*; NS, *Nitrososphaerales*.

Dissipation, transformation, and impact of iprodione and 3,5-DCA on the soil microbial community: microcosm assay in the agricultural soil. A replicate microcosm study in an agricultural soil (soil B) was subsequently employed to evaluate the universal nature of the effects induced by the application of iprodione to the soil microbial community. The dissipation of iprodione treated with the 1× dose was best described by the hockey stick (HS) model, whereas the SFO model provided adequate fit to the dissipation data for the 10× and 100× doses (Fig. S7). DT_{50} values of iprodione showed a dose-dependent trend (Table S1) and varied from 0.35 to 42.5 days

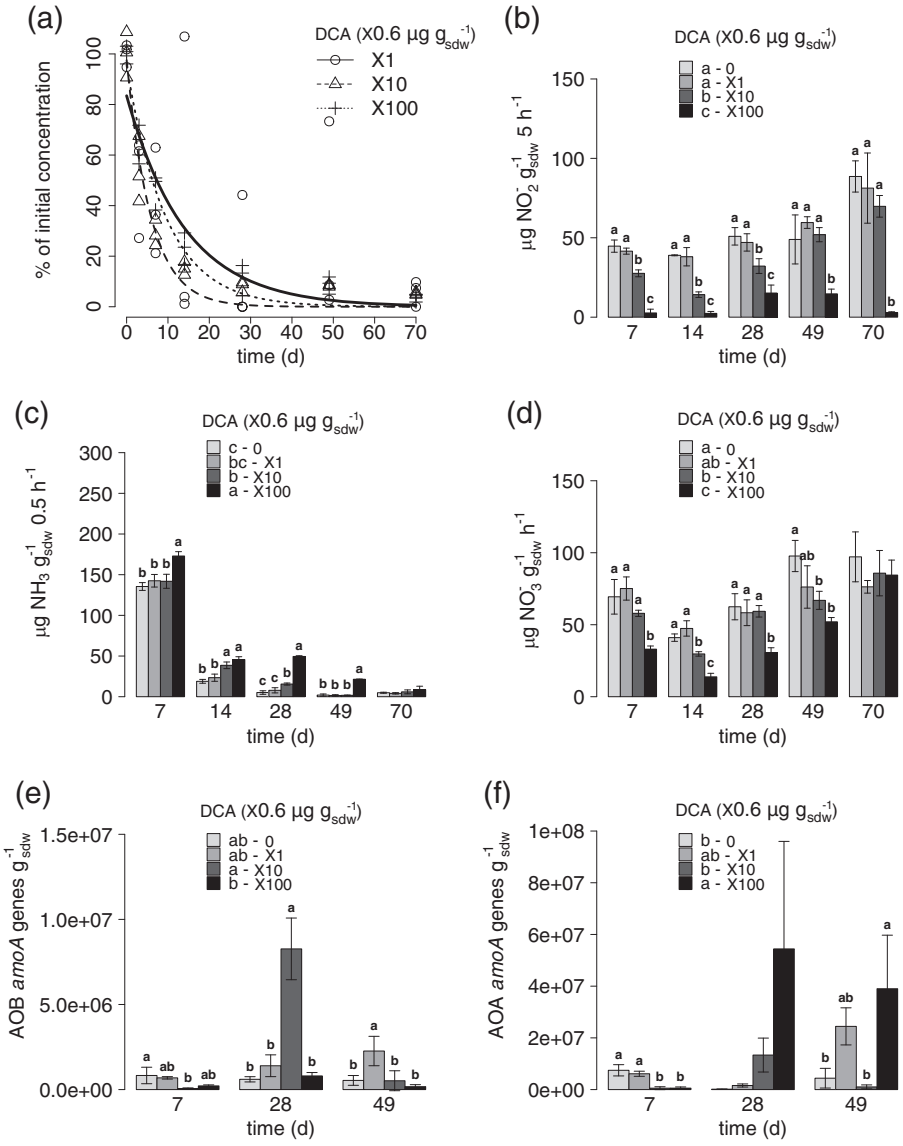

FIG 5 (a) The dissipation patterns of 3,5-dichloroaniline (3,5-DCA) in soil samples treated with 0.6, 6, and 60 μg g^{-1} and fitted to the single first-order (SFO) kinetics model. Temporal patterns of potential nitrification (PN) (b), NH_3-N (c), NO_3^--N (d), and *amoA* gene abundance of ammonia-oxidizing bacteria (AOB) (e) and archaea (AOA) (f) in samples from soil B which were treated with 0.6, 6, and 60 μg g^{-1} of 3,5-DCA. Each value is the mean of three replicates ± the standard deviation of the mean. In the bar charts, letters at the legend on the top left of each panel, where present, provide the dose main-effect groupings according to the *post hoc* analysis. Within each time point, groups designated by the same letter are not significantly different at the selected α-value levels. d, days.

in the samples treated with the 1× and 100× doses, respectively. Iprodione dissipation was accompanied by the formation of low 3,5-DCA concentrations which never exceeded 10% of the initially applied iprodione. The fungicide induced significant changes in the soil levels of NH_3-N and the abundance of the *amoA* gene of AOB and AOA; however, no clear temporal dose-dependent patterns were observed (Fig. S7).

A follow-up microcosm study was undertaken in soil B (3,5-DCA directly applied in soil) to test the hypothesis that the lack of effects on the activity of AOM in soil B was a function of the limited formation of 3,5-DCA. The dissipation of 3,5-DCA was well described by the SFO model (Fig. 5a). The dissipation of 3,5-DCA showed a weak dose-dependent pattern, with DT_{50} values ranging from 4.1 to 6.6 days in the samples treated with doses of 0.6 and 60 μg g^{-1}, respectively (Table S1). The application of

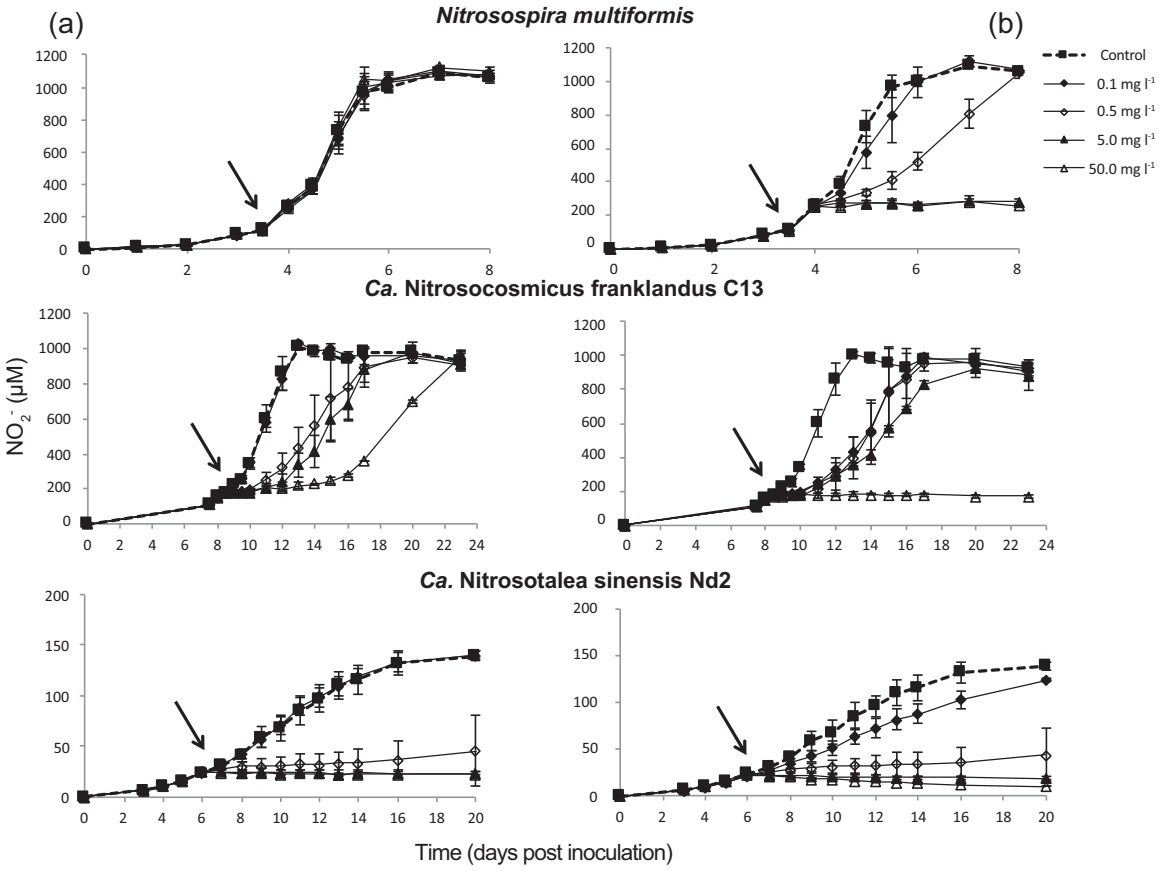

FIG 6 The effect of iprodione (a) and 3,5-dichloraniline (3,5-DCA) (b) on the ammonia oxidation activity of bacterium *Nitrosospira multiformis* and the archaea "*Ca*. Nitrosocosmicus franklandus" strain C13 and "*Ca*. Nitrosotalea sinensis" strain Nd2 in liquid cultures. Each value is the mean of three replicates ± the standard deviation of the mean. Arrows indicate the time point at which the chemicals were added in the microbial culture.

doses of 6 and 60 μg g^{-1} induced a significant reduction ($P < 0.001$) in PN, which recovered to levels similar to the level of the control by day 49 only in the samples treated with a dose of 6 μg g^{-1} (Fig. 5b). Regardless of the treatment applied, the concentrations of NH_3-N decreased with time (Fig. 5c), and this correlated with an increase in the concentrations of NO_3^--N (Fig. 5d). The application of the highest dose induced a significant increase in the concentration of NH_3-N and, reversibly, a significant decrease in NO_3^--N concentrations, which persisted until day 49. 3,5-DCA application did not induce any clear dose-dependent temporal changes in the abundance of the *amoA* gene of AOB (Fig. 5e), in contrast to AOA, whose *amoA* gene abundance was only temporarily reduced by the application of the two higher doses of 3,5-DCA (Fig. 5f).

***In vitro* assessment of the toxicity of iprodione and 3,5-DCA on AOA and AOB.** The superior toxicity of 3,5-DCA over iprodione on AOM was further tested in *in vitro* assays with selected terrestrial AOB (*Nitrosospira multiformis*) and AOA ("*Candidatus* Nitrosocosmicus franklandus" and "*Candidatus* Nitrosotalea sinensis"). Iprodione was not toxic to *N. multiformis* even at the highest concentration level tested (50 mg liter^{-1}) (Fig. 6a). In contrast, 3,5-DCA, at concentrations higher than 0.5 mg liter^{-1}, strongly inhibited the activity of *N. multiformis*, with a late recovery observed only in the cultures amended at 0.5 mg liter^{-1} (Fig. 6b). Iprodione inhibited the activity of "*Ca*. Nitrosocosmicus franklandus" at concentrations of >0.5 mg liter^{-1} although recovery was observed at all concentrations at the end of the incubation period (Fig. 6a). 3,5-DCA induced a significant reduction in the activity of "*Ca*. Nitrosocosmicus franklandus" at all concentrations; however, recovery was observed only in the cultures amended with

5 mg liter^{-1} or less (Fig. 6b). Iprodione induced a nonreversible inhibition of the activity of the acidophilic AOA "*Ca*. Nitrosotalea sinensis" at concentrations of >0.5 mg liter^{-1} (Fig. 6a), while 3,5-DCA halted the activity of "*Ca*. Nitrosotalea sinensis" at all concentration levels, and a slow recovery was observed only at 0.1 mg liter^{-1} (Fig. 6b). The inhibition profiles determined by measuring *amoA* gene abundance were concomitant with those determined via the monitoring of NO_2^- production (Fig. S8).

DISCUSSION

The application of iprodione in a meadow soil (soil A) significantly reduced the activity of enzymes involved in P (AcP, BisP, and Piro) and N (Chit and Leu) transformation (see Fig. S1 in the supplemental material). The activities of AcP, BisP, Piro, Chit, and Leu were strongly correlated with the soil concentrations of iprodione but mostly with the soil concentrations of its TP, 3,5-DCA (Fig. 3). In a series of studies, Zhang et al. (21, 24) observed a significant reduction in the activity of Bglu, AcP, alkaline phosphomonoesterase (AlkP), Leu, and Chit upon repeated applications of the recommended dose of iprodione (4 × 1.5 μg g^{-1}). These effects became evident at the later stages of the incubation (from 14 days onward) and probably reflected the gradual accumulation of iprodione and/or the formation of 3,5-DCA. However, the concentration of either compound was not measured in these studies, preventing the identification of the inhibitory mechanism. The formation of 3,5-DCA was also negatively correlated with PN although this was not in agreement with the measurements of the abundances of the *amoA* genes of AOA and AOB, which were not altered by iprodione application. The discrepancy between PN and AOA/AOB *amoA* gene abundance measurements is not surprising considering that these two methods determine different attributes (potential activity versus abundance, respectively, with partial contribution to the latter of relic DNA). PN has also been identified by Crouzet et al. (25) as the most sensitive functional descriptor of pesticide effects on soil microbial activity.

The inhibitory effect of 3,5-DCA on PN (Fig. 2) led us to examine further its potential to inhibit ammonia oxidation, which often constitutes the rate-limiting step in nitrification (26). In a replicate microcosm study with a soil from a fallow agricultural field (soil B), no adverse effects of iprodione on the nitrification activity and the abundance of the *amoA* genes of AOA and AOB were observed (Fig. S2) and this was consistent with the limited formation of 3,5-DCA in this soil (Fig. S7). The limited formation of 3,5-DCA could be attributed either to the presence in the soil of a pool of efficient 3,5-DCA-degrading microorganisms, which actively degraded 3,5-DCA as soon as it was formed, keeping its levels low during the study, or to the operation of alternative metabolic pathways where 3,5-DCA does not constitute a major TP (27). Direct application of 3,5-DCA in soil B, at concentration levels equivalent to those formed in soil A upon application of 1, 10, and 100 times the recommended dose of iprodione, induced a significant reduction in nitrification activity, as determined by measurements of PN and NH_3-N/NO_3^--N soil concentrations (Fig. 5b). Together, these findings support our initial hypothesis that 3,5-DCA, and not iprodione, drives the inhibition of nitrification in soil.

The superior toxicity of 3,5-DCA over iprodione was verified by *in vitro* assays with selected soil AOB and AOA isolates (Fig. 6). Although their sensitivities to iprodione and 3,5-DCA varied, 3,5-DCA was consistently more inhibitory than iprodione for all strains. *N. multiformis* was tolerant to iprodione compared to the two AOA whose growth and activity were inhibited, either transiently ("*Ca*. Nitrosocosmicus franklandus") or permanently ("*Ca*. Nitrosotalea sinensis"), by the higher concentrations of the fungicide. In contrast 3,5-DCA showed equivalent inhibitory levels to AOB and AOA strains, with "*Ca*. Nitrosocosmicus franklandus" being the most tolerant, followed by *N. multiformis* and "*Ca*. Nitrosotalea sinensis," which was the most sensitive. Previous *in vitro* studies with classical (i.e., nonpesticide) nitrification inhibitors have also demonstrated different levels of sensitivity of AOB (*N. multiformis*) versus AOA (*Nitrosotalea devanaterra* Nd1 and *Nitrososphaera viennensis*) (28, 29), which is perhaps a consequence of fundamental differences between AOA and AOB physiologies. The higher tolerance of *N. multiformis*

to iprodione might be a function of the notably high number of ABC transporters found in its genome which are known to be involved in organic solvents and multidrug efflux (30). Conversely, the consistently higher sensitivity of "*Ca*. Nitrosotalea sinensis" Nd2 to both compounds is reported for the first time and warrants further study. As with many other pesticides, iprodione acts at a cellular level by causing oxidative stress (31). While the exact mechanism of reactive oxygen generation by iprodione is yet unknown, it has been recently demonstrated that some AOA in culture are highly sensitive to oxidative stress, and the addition of scavenging supplements such as α-keto acids or catalase is required for growth in culture (32). Contrasting sensitivities to iprodione may therefore result from differences in physiologies of AOB and AOA with respect to oxidative stress.

In vitro assays provide a precise measure of the inherent toxicity of a compound to AOM, identify potential differences in the toxicity of a chemical to the different microbial moderators of the ammonia oxidation process (e.g., AOA versus AOB), and offer a valuable experimental platform to explore toxicity mechanisms. However, their results may deviate from soil microcosm studies due to the reduced diffusion or higher degradation of the chemicals in soil (33). To date, *in vitro* assays have not been used for the assessment of the soil microbial toxicity of pesticides although their inclusion in pesticide risk assessment testing as a conservative tier I step was proposed (34). In our study, 3,5-DCA inhibited the activity and growth of "*Ca*. Nitrosotalea sinensis" and *N. multiformis* at concentrations equivalent to the inhibitory concentrations of dicyandiamide (DCD) for the acidophilic AOA strain *Nitrosotalea devanaterra* Nd1 (28) and for *N. multiformis* (29). Our findings suggest that 3,5-DCA at concentration levels of 0.5 to 5 mg liter^{-1}, expected to be found in soil pore water upon application of the recommended dose of iprodione (assuming full conversion to 3,5-DCA), could inhibit the growth and activity of AOB and AOA strains.

We further explored the potential effects of iprodione and 3,5-DCA not only on the diversity of AOB and AOA but also on the diversity of soil bacteria and fungi (Fig. S6). Multivariate analysis of the diversity matrix obtained by amplicon sequencing suggested that iprodione and 3,5-DCA induced only subtle effects on the α-diversity of bacteria, fungi, AOB, and AOA. In contrast, significant effects on the β-diversity of the bacterial and fungal communities were observed. The changes found were strongly correlated with the soil levels of 3,5-DCA and not iprodione. The abundances of *Actinobacteria*, especially of the genera *Ilumatobacter* and *Solirubrobacter*, and of alphaproteobacteria of the family *Hyphomicrobiaceae* were negatively correlated with 3,5-DCA formation (Fig. 4). Previous studies have also reported a sensitivity of these bacterial phyla to high soil concentrations of pesticides (35) and other organic pollutants (36). The positive correlation of *Arthrobacter* and *Xanthomonadaceae* with the soil levels of iprodione is in line with the assignment of all the currently known iprodione-degrading soil bacteria to the genus *Arthrobacter* (37, 38) and the well-documented role of members of the family *Xanthomonadaceae* in the degradation of pesticides (39) and antibiotics (40). Changes in fungal community structure were observed (Fig. S6), with *Sordariomycetes* being positively correlated with iprodione soil concentrations (Fig. 4), in agreement with Zhang et al. (20), who reported an enrichment of OTUs belonging to families of *Sordariomycetes*, including *Cephalothecaceae*, *Hypocreaceae*, and *Cordycipitaceae*. Conversely, 3,5-DCA formation favored fungi of the family *Lasiosphaeriaceae* and reduced the abundance of yeasts of the family *Pichiaceae*. The former family encompasses coprophilous fungi like *Podospora* and *Zopfiella* which are commonly found in manured soils (41), like the meadow soil studied. The *Pichiaceae* include yeasts with applications in agriculture (plant growth promoters or biocontrol agents) (42), food technology (alcohol fermentation), and biotechnology (43).

The AOB and AOA communities in the meadow soil were dominated by members of the genus *Nitrosospira* and the lineage *Nitrososphaerales* (clades γ, δ, and ε), respectively (Fig. S4 and S5), which are ubiquitous in the soil environment (44, 45), with *Nitrososphaerales* clades γ and δ representing over 65% of the currently reported soil/sediment AOA (23). Despite the inhibitory effects on soil nitrification, the β-diversity of AOB and AOA was not significantly altered by iprodione and 3,5-DCA (Fig.

S6), whose soil concentrations were positively correlated with rare OTUs of the AOB community (Fig. 4). In contrast, only dominant AOA OTUs were responsive to iprodione and 3,5-DCA. These results might be an indication of the crucial functional role of the rare and dominant members of the AOB and AOA communities, respectively. Alternatively, other AOM like the comammox bacteria, not considered in the current study, might have an important functional role in the nitrification in the soil studied although their ecological role in terrestrial ecosystems is still not well defined (46).

Conclusions. To date, most of the studies that have investigated the impact of pesticides on soil microorganisms have attributed observed effects exclusively to the parent compound while the contribution of TPs was largely overlooked (47, 48). This is particularly true for iprodione, whose structural and functional effects on the soil microbial community (19, 20, 49), and particularly on AOM (50), were fully attributed to the parent compound. We provide strong evidence that 3,5-DCA, the main TP of iprodione in soil, is responsible for the significant reduction in the activity of AOM and the effects on the β-diversity of bacteria and fungi. Our findings have important practical implications for pesticide environmental risk assessment since they (i) provide strong evidence for the sensitivity of AOM to pesticides, in line with a series of previous studies (4, 6, 9–11, 25, 47), reinforcing their potential as indicators of the soil microbial toxicity of pesticides, and (ii) demonstrate that the TPs of pesticides could have a higher intrinsic soil microbial toxicity than their parent compounds; hence, assessment of their soil microbial toxicity should be an integral part of environmental risk analysis.

MATERIALS AND METHODS

Soils and chemicals. The impact of iprodione and 3,5-DCA was assessed in two different top-soils (0 to 20 cm): (i) a Cambisol loam (pH 6.9; organic carbon content, 2.98%) collected from a meadow in northern Italy (Fontidella Gaverina, 45°27′55.69″N, 9°38′20.05″ E; soil A) and (ii) a Cambisol clay loam (pH 7.6; organic carbon content, 1.1%) collected from a fallow agricultural field of the Hellenic Agricultural Organization-Demeter in Larissa, Greece, with no recent history of pesticide application (39°63′27″N, 22°36′74″E, soil B). Both soils were collected from the top 20 cm according to a protocol of the International Organization for Standardization (https://www.iso.org/standard/43691.html) for collection and handling of samples. Upon collection, soils were homogenized, partially air dried, sieved (2-mm pore size), and stored at 4°C for a week before use. Iprodione and 3,5-DCA analytical standards (>97% purity) were used for analytical purposes and *in vitro* tests, while a commercial formulation of iprodione (Rovral 50%WP) and the analytical standard of 3,5-DCA were used in microcosm tests.

Soil microcosm experiments. Four 1-kg (each) subsamples of soils A and B were pretreated with 2.5 ml of a 0.5 M solution of $(NH_4)_2SO_4$ (corresponding to 154 mg N kg^{-1} soil dry weight). Samples were left to equilibrate overnight and then spiked with appropriate volumes of aqueous solutions of iprodione corresponding to application of 1, 10, and 100 times the recommended dose and soil concentrations of 2, 20, and 200 μg of iprodione g^{-1} soil, respectively. The fourth soil sample received the same amount of water without iprodione as a control. The soil moisture content was adjusted to 40% of the water-holding capacity. Bulk soil samples were divided into 50-g subsamples which were placed in aerated plastic bags and incubated in the dark at 25°C. At regular intervals, triplicate samples per treatment were sampled and analyzed for iprodione and 3,5-DCA residues or used for determination of enzyme activity, PN, or DNA extraction.

To further explore the microbial toxicity of 3,5-DCA (in soil B), NH_4^+-amended soil samples were treated with 3.3 ml of methanolic solutions of 3,5-DCA at concentrations of 100, 1,000, and 10,000 μg ml^{-1}, resulting in final soil 3,5-DCA concentrations of 0.6, 6, and 60 μg g^{-1} soil, respectively. These corresponded to the maximum concentrations of 3,5-DCA formed in soil by the application of 1, 10, and 100 times the recommended dose of iprodione in soil A. The fourth subsample received the same volume of methanol without 3,5-DCA as an untreated control. Samples were left for 1 h to allow evaporation of methanol and were then processed as described above. Immediately after 3,5-DCA application and at regular intervals thereafter, triplicate microcosms were sampled per treatment and analyzed for 3,5-DCA, NO_3^--N, and NH_4^+-N concentrations or processed for DNA extraction and PN measurements.

Pesticide residue and NH_3/NO_3^--N analysis. Iprodione and 3,5-DCA residues were extracted from soil as described by Vanni et al. (51) with the sole modification that the final acetonitrile extract was dried under a flow of nitrogen, and the samples were resuspended in acetone containing the internal standard anthracene-d10 (*m/z* 188). Extracts were analyzed in an Agilent 6890 gas chromatograph-mass spectrometry (GC-MS) system equipped with an Agilent Technologies 5973 series mass selective detector. The column used was a Supelco SLB-5 MS type (30 m by 0.25-mm internal diameter by 0.25-μm film thickness). The carrier gas was high-grade helium used at a constant flow rate of 1.0 ml min^{-1}. Injection temperature was 250°C, with an injection volume of 1 μl and a 0.5-min purge time. The GC-MS oven temperature was maintained at 70°C for 2 min and then increased at a rate of 10°C min^{-1} until 230°C. This temperature was maintained for 3 min and finally increased at a rate of 25°C min^{-1} until it reached 280°C, and it remained constant for 6 min. The detector temperatures were 150°C (MS quadrupole) and 230°C (MS source). The compounds were identified in total ion-monitoring mode, at *m/z* 161 for 3,5-DCA

TABLE 1 Primer sequences used for PCR amplification for multiplexed sequencing

Target gene	Primer name	Sequence[a]	Thermal cycling conditions[b]	Reference
Bacterial 16S rRNA	343f	NNNNNNN**TA**TACGGRAGGCAGCAG	94°C for 30 s, 50°C for 30 s,	64
	802r	TACNVGGGTWTCTAATCC	72°C for 30 s (25 + 7 cycles)	
Fungal ITS1	ITS-1	NNNNNNN**AA**TCCGTAGGTGAACCTGCGG	94°C for 30 s, 56°C for 30 s,	84
	ITS-2	GCTGCGTTCTTCATCGATGC	72°C for 60 s (28 + 7 cycles)	
AOB *amoA*	amoA-1f	NNNNNNN**AA**GGGGTTTCTACTGGTGGT	94°C for 30 s, 54°C for 30 s,	60
	amoA-2r	CCCCTCKGSAAAGCCTTCTTC	72°C for 60 s (25 + 5 cycles)	
AOA *amoA*	amoA-310f	NNNNNNN**GG**TGGATACCBTCWGCAATG	94°C for 30 s, 54°C for 30 s,	85
	amoA-529r	GCAACMGGACTATTGTAGAA	72°C for 60 s (25 + 5 cycles)	

[a]The sample index (consecutive Ns) and linker (bold letters) prior to the extension bases in the forward primer are indicated.
[b]The first number in parentheses indicates the number of cycles performed in the fist PCR where the unindexed primers were used, while the second number indicates the additional cycles performed in the sample indexing PCR step.

and *m/z* 187 for iprodione. Soil levels of NH_4^+-N and NO_3^--N were determined as described by Kandeler and Gerber (52) and Doane and Horwáth (53), respectively.

***In vitro* assays.** The inhibitory effects of iprodione and 3,5-DCA on soil AOB (*N. multiformis*) and AOA ("*Ca*. Nitrosotalea sinensis" strain Nd2 and "*Ca*. Nitrosocosmicus franklandus" strain C13) were investigated in liquid batch cultures. "*Ca*. Nitrosocosmicus franklandus" was isolated from a Scottish agricultural soil with pH 7.5 (54) and "*Ca*. Nitrosotalea sinensis" was from a Chinese acidic paddy soil (pH 4.7) (55, 56). All strains were grown aerobically in the dark without shaking. *N. multiformis* was grown at 28°C in Skinner and Walker's medium (57) (pH of 7.5). AOA strains were incubated at 35°C in medium supplemented with 1 mM NH_4^+. "*Ca*. Nitrosocosmicus franklandus" C13 was cultured in HEPES-buffered modified fresh water medium (pH 7.5) (54), while "*Ca*. Nitrosotalea sinensis" was grown in an acidic morpholineethanesulfonic acid (MES)-buffered (pH 5.2) freshwater medium (58). For each treatment, triplicate 100-ml Duran bottles containing 50 ml of growth medium were inoculated with a 1 or 2% (vol/vol) transfer of AOB and AOA culture, respectively, in exponential growth. Iprodione and 3,5-DCA were added to the cultures at four concentration levels: 0.1, 0.5, 5, and 50 mg liter^{-1} in 0.1% (vol/vol) dimethyl sulfoxide (DMSO) once exponential growth was observed. Triplicate cultures amended with 0.1% (vol/vol) DMSO without iprodione and 3,5-DCA served as controls. The effects of iprodione and 3,5-DCA on the growth and activity of AOM were measured regularly via determination of the *amoA* gene abundance and NO_2^- levels (59), respectively. DNA was extracted from a cell pellet obtained from 2-ml aliquots of the microbial cultures using a tissue DNA extraction kit (Macherey-Nagel, Germany).

DNA extraction from soil. DNA was extracted from ~500 mg of soil using a FastDNA Spin kit for soil and a FastPrep instrument (MP Biomedicals, USA) and quantified using a Qubit fluorometer with a Quant-iT HS double-stranded DNA (dsDNA) assay kit (Invitrogen, USA).

qPCR analysis of functional genes. The abundances of AOB and AOA *amoA* genes were determined with primers amoA1F-amoA1R (60) and CrenamoA23f-CrenamoA616r (61), respectively, as described by Rousidou et al. (62), and the *soxB* gene using primers soxB_710f-soxB1184R as described in Tourna et al. (63). Quantitative PCR (qPCR) amplification efficiencies were between 85 and 102%, with R^2 values of ≥0.993. Standard curves were obtained using serial dilutions of plasmid vectors containing amplicons of nearly full-length target genes.

Community structure analysis of bacteria, fungi, and AOM. Microbial diversity analysis for total bacteria, fungi, AOB, and AOA was performed on samples from all treatments collected at 0, 15, and 42 days via multiplex amplicon sequencing in an Illumina MiSeq sequencer generating 300-bp paired-end reads as described previously (64). Primers, primer-indexing sequences, PCR conditions, and programs are shown in Table 1 and in Table S4 in the supplemental material. Sequence screening, alignment to reference databases, and generation of OTU matrices for bacteria, AOB, and AOA were performed with mothur, version 1.36.1 (65), while the fungal ITS1 amplicon screening was performed with USEARCH, version 9 (66). Bacterial 16S rRNA gene amplicon sequences were obtained through assembly of the read pairs subsequently aligned against the Silva, version 128, bacterial database (67, 68). AOB/AOA *amoA* amplicons were compared with the alignments of Abell et al. (22) and Alves et al. (23), respectively, for generating OTUs. Sequences were then clustered into groups differing by 1% in identity. Chimeric amplicons were identified and removed using UCHIME, version 4.2 (69). Sequence distances were calculated for the aligned sequences, while hierarchical clustering using the average linkage algorithm (70) was performed for identifying OTUs at 0.02 sequence distances. Sequence divergences of 0.02 and 0.03 were used for bacteria and ammonia oxidizer OTU definition, respectively. Fungal ITS amplicons were clustered with the USEARCH, version 9, implemented with the UCLUST algorithm (66) using the UNITE (71), version 7.2, database ITS reference data set at a 0.03-distance OTU definition. The most abundant sequences of each OTU were selected as representatives and were classified according to the Ribosomal Database Project (RDP) with the naive Bayesian classifier module residing in mothur, version 1.36.1, for bacteria and the UNITE database for fungi with the USEARCH, version 9, native SINTAX algorithm. AOB and AOA *amoA* gene OTU representative sequences were placed in the previously curated phylogenies of Abell et al. (22) and Alves et al. (23), respectively. This was performed using the parsimony-based phylogeny-aware short-read alignment approach of PaPaRa, version 2.4, combined with a maximum likelihood evolutionary placement algorithm as implemented in RAxML, version 8.2.4 (72–74).

Soil enzyme activities and PN measurements. The activities of AcP (EC 3.1.3.2), AlkP (EC 3.1.3.1), BisP (EC 3.1.4.1), and Piro (EC 3.6.1.1), involved in P cycling, arylsulfatase (AryS; EC 3.1.6.1) involved in S cycling, Chit (EC 3.2.1.52) and Leu (EC 3.4.11.1), involved in N cycling, and Bglu (EC 3.2.1.21), involved in C cycling, were determined using fluorescent 4-methyl-umbelliferyl and 7-amino-4-methyl coumarin (for Leu) linked to appropriate enzyme substrates and determined in a fluorometric plate reader system as described by Karas et al. (9). PN was determined by the method of Kandeler (75). Briefly, 5-g soil samples were amended with 20 ml of 1 mM $(NH_4)_2SO_4$ and 0.1 ml of 1.5 M $NaClO_3$ and incubated under constant agitation at 20°C for 5 h, while triplicate control samples were treated in the same way and incubated at −20°C for the same period. At the end of the incubation period, NO_2^- was extracted from all samples with 2 M KCl. The extract (5 ml) was amended with 3 ml of 0.19 M NH_4Cl and 2 ml of a colorimetric indicator prior to final determination of its adsorption at 520 nm. The PN in the soil samples was then determined with an external calibration curve prepared by measurement of the adsorption of a series of $NaNO_2$ solutions.

Data analysis. The SFO kinetic model or the biphasic model HS was used to calculate the soil dissipation kinetics of iprodione and 3,5-DCA (76). The SFO kinetic model is based on the assumption that the change in a chemical's concentration with time (*dC/dt*) is directly proportional to its concentration at this time. The HS model involves two sequential first-order degradation phases with different rates (k_1 and k_2) and having a breakpoint between them (t_b) (24). The goodness of fit was assessed using a χ^2 test (<15%, for an α of 0.05), visual inspection, and the distribution of residuals.

Microbial functional measurements, *in vitro* assays, and α-diversity index data were analyzed by two-way analysis of variance (ANOVA) and associated *post hoc* tests. RDA of the PN and enzyme activities with the ln-transformed soil concentrations of iprodione and 3,5-DCA as explanatory variables was employed to identify associations between compounds and the effects observed. The OTU matrices of bacteria, fungi, AOA, and AOB were used to assess the impact of iprodione and 3,5-DCA on the α- and β-diversity values. A-diversity indices used included Good's coverage estimate (77), the Shannon index, the inverse Simpson index (78), observed richness (*S*), and ACE (abundance-based coverage estimator) richness estimation (79). β-Diversity analysis included hierarchical clustering using the Bray-Curtis dissimilarity and RDA on the Hellinger-transformed matrices (80–82). Correlation tests between the measured concentrations of iprodione and 3,5-DCA in soil and the OTU-assigned sequence counts identified possible effects of the two chemicals on the microbial community members. All statistical analyses were performed with R, version 3.3.2, software (83). More details on data analysis are provided in the supplemental material.

Accession number(s). The sequence data are publicly available in the National Center for Biotechnology Information (NCBI) database under BioProject accession number PRJNA472261.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01536-18.

SUPPLEMENTAL FILE 1, PDF file, 1.0 MB.

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